Heat-Shock Protein 70 (Hsp70) as a Biochemical Stress Indicator: an Experimental Field Test in Two Congeneric Intertidal Gastropods (Genus: *Tegula*)

LARS TOMANEK AND ERIC SANFORD

Abstract. Although previous studies have demonstrated that heat-shock protein 70 (Hsp70) can be induced by environmental stress, little is known about natural variation in this response over short time scales. We examined how Hsp70 levels varied over days to weeks in two intertidal snail species of the genus *Tegula*. Sampling was conducted both under naturally changing environmental conditions and in different vertical zones on a rocky shore. The subtidal to low-intertidal *T. brunnea* was transplanted into shaded and unshaded mid-intertidal cages to assess temporal variation in Hsps under conditions of increased stress. For comparison, the low to mid-intertidal *T. funebralis* was transplanted into mid-intertidal cages, within this species' natural zone of occurrence. Snails were sampled every 3 to 4 days for one month, and endogenous levels of two Hsp70-kDa family members (Hsp72 and Hsp74) were quantified using solid-phase immunochemistry. Following periods of midday low tides, levels of Hsps increased greatly in transplanted *T. brunnea* but not in *T. funebralis*. Levels of Hsps increased less in *T. brunnea* transplanted to shaded cages than to unshaded cages, suggesting that prolonged emersion and reduction in feeding time *per se* are factors that are only mildly stressful. Upregulated levels of Hsps returned to base levels within days. In unmanipulated snails collected from their natural zones, Hsp levels showed little change with thermal variation, indicating that these species did not experience thermally stressful conditions during this study. However, under common conditions in the mid-intertidal zone, Hsp70 levels reflected the different thermal sensitivities of the physiological systems of these two species.

Introduction

The synthesis of heat-shock proteins (Hsps) is induced when environmental variation perturbs an organism's physiological system to the extent that its proteins denature. Under such environmental conditions Hsps and other molecular chaperones stabilize denaturing proteins, refold reversibly denatured proteins, and facilitate the degradation of irreversibly denatured proteins (Lindquist, 1986; Lindquist and Craig, 1988; Parsell and Lindquist, 1994; Feige *et al.*, 1996; Frydman, 2001; Hartl and Hayer-Hartl, 2002). Numerous studies have investigated the relationship between Hsp synthesis and various potential stress factors; however, very few studies have investigated the variation of Hsp levels under varying natural conditions (for review see Feder and Hofmann, 1999). Even fewer studies have investigated short-term variation in Hsp levels (*e.g.*, hours, days to weeks) in response to variable physical conditions in the field (Hofmann and Somero, 1995; Nakano and Iwama, 2002). A comprehensive understanding of such a time course of variation in Hsp levels under natural conditions is needed to interpret Hsp levels from field-collected organisms and therefore to evaluate stress under natural conditions. Furthermore, predictions about the ecological role of the heat-shock response that are made from laboratory comparisons of species that occupy widely varying thermal environments have not been tested under natural conditions.

Our study focuses on Hsp variation in response to physical stress in intertidal organisms. Physical factors, in particular temperature, play an important role in setting the upper limits to the vertical distribution range of intertidal organisms and confine them to distinct bands on the shore

with species-specific upper and lower vertical limits (see reviews in Benson, 2002; Tomanek and Helmuth, 2002). This view is supported by numerous laboratory studies demonstrating that physiological resistance to physical conditions is greater in species that live at higher tidal heights (Newell, 1979; Somero, 2002).

Intraspecific variation in the expression of Hsps has been found in intertidal species in association with seasonal acclimatization (Dietz and Somero, 1992; Hofmann and Somero, 1995; Roberts *et al.,* 1997; Chapple *et al.,* 1998; Buckley *et al.,* 2001), laboratory acclimation (Hofmann and Somero, 1996a; Roberts *et al.,* 1997; Tomanek and Somero, 1999, 2000, 2002), competition for space (Rossi and Snyder, 2001), food availability and wave exposure (Dahlhoff *et al.,* 2001), and microhabitat (Helmuth and Hofmann, 2001). Interspecific differences in the heat-shock response, especially among congeneric species, often correlate positively with thermal extremes in the environment (Sanders *et al.,* 1991; Dietz and Somero, 1993; Hofmann and Somero, 1996a; Tomanek and Somero, 1999, 2000, 2002; Nakano and Iwama, 2002; Tomanek, 2002). Some of these studies have shown how Hsp levels vary over hours in response to thermal stress under natural (Hofmann and Somero, 1995, 1996b; Nakano and Iwama, 2002) as well as laboratory conditions (Tomanek and Somero, 2000). To our best knowledge, nothing is known about the variation of Hsp levels in response to changing natural conditions over days to weeks. Furthermore, whether interspecific differences in the heat-shock response limit a species' thermal niche and contribute to setting its vertical distribution range within the intertidal zone has not been tested under natural conditions.

This study focuses on two herbivorous gastropod species of the genus *Tegula* that occupy distinct vertical zones on the shore: *T. brunnea* (Philippi, 1848), common in the subtidal to low-intertidal zone, and *T. funebralis* (Adams, 1855), common in the low- to mid-intertidal zone (Riedman *et al.,* 1981; Watanabe, 1984). Our previous laboratory work suggested that heat stress might prevent the low-intertidal *T. brunnea* from occupying the mid-intertidal zone (Tomanek and Somero, 1999, 2000, 2002). Thus, we predicted that *T. brunnea* transplanted upward into the mid-intertidal zone would express increased levels of Hsp70 in its new thermal environment. Laboratory results (Tomanek and Somero, 1999) also suggested that the mid-intertidal *T. funebralis* would activate the heat-shock response frequently in its natural zone of occurrence due to the temperature extremes and fluctuations that characterize the mid-intertidal zone. In this study we test these predictions by following the time course of expression of two Hsp70 isoforms (Hsp72 and Hsp74) over a month-long sampling period in specimens transplanted from the low-intertidal into the mid-intertidal zone, and in control individuals collected from their natural vertical zones.

This combination of ecological and molecular approaches thus attempts to comprehensively test how Hsp levels vary over time in response to the changing physical conditions within and beyond a species' natural thermal zone.

Materials and Methods

Study organisms and distribution patterns

The two *Tegula* congeners used in this study differ in their biogeographic and vertical distributions. *Tegula brunnea* inhabits the subtidal to low-intertidal zones of the eastern Pacific Ocean from Cape Arago, Oregon (43° 25′N) to the Channel Islands, California (34° 00′N) (Abbott and Haderlie, 1980; Riedman *et al.,* 1981; Watanabe, 1984). *Tegula funebralis* is found in the low- to mid-intertidal zone and has a wider latitudinal range, from Vancouver Island, British Columbia, Canada (48° 25′N), to central Baja California, Mexico (28° 00′N) (Abbott and Haderlie, 1980; Riedman *et al.,* 1981).

Experimental design

Field experiments were conducted at Hopkins Marine Station of Stanford University in Pacific Grove, California (36° 36′N, 121° 54′W). To test the role of temperature in setting the upper vertical limit of the subtidal to low-intertidal *T. brunnea,* we transplanted this species above its natural zone of occurrence. Snails (shell diameter = 20–25 mm, marked with yellow nail polish) were placed into stainless steel cages (20 × 20 cm wide and 5 cm high; 316 stainless steel wire cloth with a 3-mm opening size) that were positioned at similar heights in the mid-intertidal zone (+0.64 ± 0.12 m above mean lower low water). These enclosures had no bottom and thus allowed snails to move over the rock surface and feed on the algal species present naturally. Cages were either unshaded (sun-exposed treatment) or shaded by covering them with plastic mesh (two layers of a black polyethylene mesh with a 3.8-mm opening size). Each treatment included seven cages, with 10 snails per cage.

In addition, we transplanted *T. funebralis* from the mid-intertidal zone into unshaded mid-intertidal cages ($n = 7$) positioned at the same tidal height. This treatment served two purposes. First, it allowed us to compare the thermal stress of *T. brunnea* transplanted into the mid-intertidal zone (above its natural upper limit) to that of similarly caged *T. funebralis,* the natural inhabitant of the mid-intertidal zone. Secondly, it allowed us to test for caging artifacts by comparing caged mid-intertidal *T. funebralis* to unmanipulated snails found naturally in that zone.

Single snails were collected from each cage and replaced with an unmarked specimen (to avoid changes in snail density during the experiment) every 3rd or 4th day over a month-long sampling period (31 March to 1 May 2000; see Figs. 1 and 2). To evaluate the response of snails in their

native thermal environment, we also collected unrestricted snails from the mid-intertidal (*T. funebralis*) and the shallow subtidal zone (*T. brunnea;* specimens were always submerged before and during collection). All collections were made within 45 min of low tide to minimize any physiological variation that might be related to endogenous tidal rhythms. Snails were frozen immediately on dry ice following collection and kept at −70 °C until further processing. To obtain a record of *Tegula* body temperature, temperatures in gelatin-filled snail shells were recorded inside mid-intertidal cages and outside (on adjacent rocks) by a StowAway XTI temperature data logger (Onset Computer Corp, Pocasset, MA). For further details on this method, see Tomanek and Somero (1999). Because of equipment failure, only one complete record, from a *T. funebralis* shell attached to open rock adjacent to a mid-intertidal cage, was obtained from the six data loggers installed. Temperatures shown (Figs. 1 and 2) therefore represent the thermal variation of field-acclimatized *T. funebralis* from the mid-intertidal zone. Immediately following the experiment (and discovery of the equipment failure), additional data loggers were deployed to characterize the difference among our treatments. During midday low tides, temperatures within unshaded cages were typically 3–6 °C cooler than on the open rock outside the cage, whereas temperatures in shaded cages were an additional 2–5 °C cooler than in cages without shades.

Tissue preparation

Gill tissue was dissected from whole snails that were thawed under conditions that do not induce heat shock (13 °C) and immediately placed in 200 μl (*T. funebralis,* 15.0 to 25.0 mg wet weight) or 300 μl (*T. brunnea,* 30.0 to 45.0 mg wet weight) of homogenization buffer (32 mmol l^{-1} Tris-HCl, pH 7.5 at 4 °C, 2% (w/v) SDS, 1 mmol l^{-1} EDTA, 1 mmol l^{-1} Pefabloc (Boehringer Mannheim), 10 μg ml^{-1} pepstatin, and 10 μg ml^{-1} leupeptin). Tissues were incubated for 5 min at 100 °C and homogenized. The procedure was repeated and homogenates were centrifuged at 15,800 × *g* for 15 min. The supernatant was removed and stored at −70 °C. Protein concentrations were determined using the Micro-BCA assay (Pierce) according to the manufacturer's instructions.

Gel electrophoresis and immunodetection (Western) protocol

In general, we followed the procedure described in Tomanek and Somero (2002). Briefly, proteins were separated electrophoretically and subsequently transferred onto nitrocellulose membranes (Nitrobind, Schleicher and Schuell) in transfer buffer (25 mmol l^{-1} Tris-base, 0.193 mol l^{-1} glycine, 20% methanol (v/v), pH 8.3 at 20 °C). After membranes were dried overnight they were treated with blocking buffer (25 mmol l^{-1} Tris-HCl, pH 7.5 at 20 °C, 150 mmol l^{-1} NaCl, 0.1% (v/v) Tween, 0.02% (w/v) Thimerosol, 5% (w/v) nonfat dried milk) for 1 h, subsequently washed with Tris-buffered saline (TBS; 25 mmol l^{-1} Tris-HCl, pH 7.5 at 20 °C, 150 mmol l^{-1} NaCl), and then incubated with a solution of a monoclonal rat antibody (IgG) against Hsp70 (clone 7.10; Affinity BioReagent, MA3-001; 1:2500 dilution of Hsp70 antibody in buffer A (BA): TBS, 2.5% (w/v) bovine serum albumin in TBS) for 1 h. After washing the membranes, we incubated them for 30 min with a rabbit-anti-rat bridging antibody (IgG) solution (1:2000 dilution in BA; Vector, AI-4000), followed again by several washing steps. Finally, we incubated membranes with a horseradish-peroxidase protein A solution (1:5000 dilution in BA; Bio-Rad) for 30 min. Membranes were washed and overlaid with a solution of enhanced chemiluminescent (ECL) reagent (Amersham Pharmacia) according to the manufacturer's instructions for 1 min. Under dark room conditions, we exposed membranes onto pre-flashed Hyperfilm (Amersham Pharmacia) for 5, 10, 20, 30, and 50 min after ECL treatment to obtain various exposures that were in the linear range of detection. All samples were run at least twice.

Image analysis and quantification of expression of heat-shock proteins

Film images were scanned on a densitometer (Sharp JX-330) and the digitized images were analyzed with image analysis software (ImageMaster 1D, ver. 2.01, Pharmacia) to quantify band intensities of the two Hsp70 isoforms, one with a molecular mass of about 72 kDa (Hsp72), the other of about 74 kDa (Hsp74). We express band intensities relative to a known amount of a bovine heat-shock cognate 70 (80 ng; StressGen, SPP-750) to account for variation among Western blots.

Statistical analysis

Variation in Hsp72 and Hsp74 was compared using a two-factor analysis of variance (ANOVA) with experimental treatments and sampling days as the main effects. We conducted *post hoc* comparisons of all five treatment groups (Student-Newman-Keuls test) within each sampling day separately. To calculate the critical value, we used the appropriate Studentized range statistic ($\alpha = 0.95$; m = number of means; df = degrees of freedom of the error term from the ANOVA) and an adjusted n value (n_0) to account for unequal sample sizes among the means using the following equation:

$$n_0 = \frac{1}{a-1}\left(\sum^{a} n_i - \frac{\sum^{a} n_i^2}{\sum^{a} n_i}\right)$$

with "a" the total number of means compared ($a = 50$) and "n_i" the sample size for each mean. Variances were not heterogeneous (Cochrane's test, $P > 0.05$), and therefore there was no need to transform the data.

A cross-correlation analysis (MatLab Software) compared average, minimum, maximum daily temperatures as well as daily temperature range with endogenous levels of Hsp72 and Hsp74 over the entire sampling time for field-acclimatized mid-intertidal *T. funebralis* only.

Results

Data logger records indicate that the body temperature of mid-intertidal *Tegula* varied dramatically with tidal cycle and date over the course of this experiment (Figs. 1A and 2A). The three panels B, C, and D (Figs. 1 and 2) show the endogenous levels of Hsp72 and Hsp74 over the month of sampling.

Variation of Hsp70 in Tegula brunnea *transplanted above its natural limit*

Specimens of *T. brunnea* transplanted into unshaded cages in the mid-zone often showed dramatically elevated levels of both Hsp72 and Hsp74 relative to control individuals collected from the shallow subtidal zone (for statistical results, see Figs. 1B and 2B). The overall response of both Hsps was very similar. Moreover, these elevated levels of Hsps seen in transplanted individuals were correlated with environmental factors: 3 days on which Hsp72 and Hsp74 levels were 2 to 4 times higher in sun-exposed mid-intertidal snails than in control snails from the shallow subtidal were preceded by periods of 2 or more days of midday low tides that greatly raised body temperatures for 1–4 h (3 and 13 April and 1 May 01). However, on 21 April, sun-exposed specimens of *T. brunnea* showed 6 times higher endogenous levels of Hsp72 and Hsp74 than control animals, but maximal daily temperatures during the preceding 4 days were relatively low compared to other time periods.

In addition, individuals of *T. brunnea* that were transplanted into shaded mid-zone cages showed elevated levels of Hsps only slightly more often than control snails from the shallow subtidal, and to a greater degree in the case of Hsp74 (*e.g.,* 10 April and 24 April) than in Hsp72. In contrast, sun-exposed individuals often showed greater Hsp levels relative to their shaded conspecifics that were transplanted into the mid-zone, with Hsp72 levels almost always being higher in the sun-exposed *T. brunnea* (Fig. 1B).

Time course of Hsps in transplanted and field-acclimatized Tegula funebralis

We quantified the time course of Hsp levels in specimens of *T. funebralis* from the mid-intertidal zone that were either experimentally caged (sun-exposed) or unrestricted (field-acclimatized) to address three issues. First, we tested for caging artifacts by comparing caged and uncaged snails within the same zone. Second, we tested the prediction that the mid-intertidal *Tegula* congener would activate the heat-shock response more frequently than the shallow subtidal congener due to their differing thermal environments and heat-shock responses (Tomanek and Somero, 1999, 2000). Third, we compared the response to thermal stress in the two temperate *Tegula* congeners that occupy different tidal heights under "common garden" conditions (see below).

Unrestricted individuals of *T. funebralis* were collected in crevices next to the unshaded cages. The two groups did not differ until the last week in April, when Hsp72 levels were higher in sun-exposed caged snails than in field-acclimatized snails (Fig. 1C). Preceding this time period, peak temperatures from data loggers were relatively low, but they increased greatly with the onset of a 10-day period of early to midday low tides. Hsp74 levels showed a similar pattern, but in addition, field-acclimatized individuals showed higher levels than caged snails from 10 to 13 April (Fig. 2C). Thus, caged specimens of *T. funebralis* were apparently more thermally stressed than their unrestricted conspecifics during the last week of the study, perhaps because caging limited the access of snails to shaded and moist microhabitats.

With time and changing temperatures, Hsp72 levels varied little until they increased in caged but not in unrestricted individuals (Fig. 1C). Although Hsp74 levels showed greater temporal variability, the resulting changes were still within the range of variation observed for shallow subtidal *T. brunnea* (Fig. 2B, C—field samples). Neither Hsp72 nor Hsp74 showed any correlations with any of the temperature variables (cross-correlation analysis, $P > 0.05$). These results suggest that mid-intertidal *T. funebralis* does not elevate levels of Hsps more often than *T. brunnea* does under the less thermally variable conditions of the shallow subtidal.

Interspecific comparisons of Tegula *in the mid-zone*

Transplanting both species to unshaded cages in the mid-intertidal zone allowed us to compare their responses to thermal stress under common garden conditions. Whereas transplanted specimens of *T. brunnea* responded to thermal stress, specimens of *T. funebralis* caged in the mid-zone (their natural zone of occurrence) changed little (Figs. 1D and 2D). Elevated levels of both Hsps indicate a response to thermal variation during time periods of midday low tides in *T. brunnea* (see above for details). Although base levels of Hsp72 in sun-exposed *T. brunnea* were close to levels found for *T. funebralis,* Hsp74 levels were, regardless of the tidal

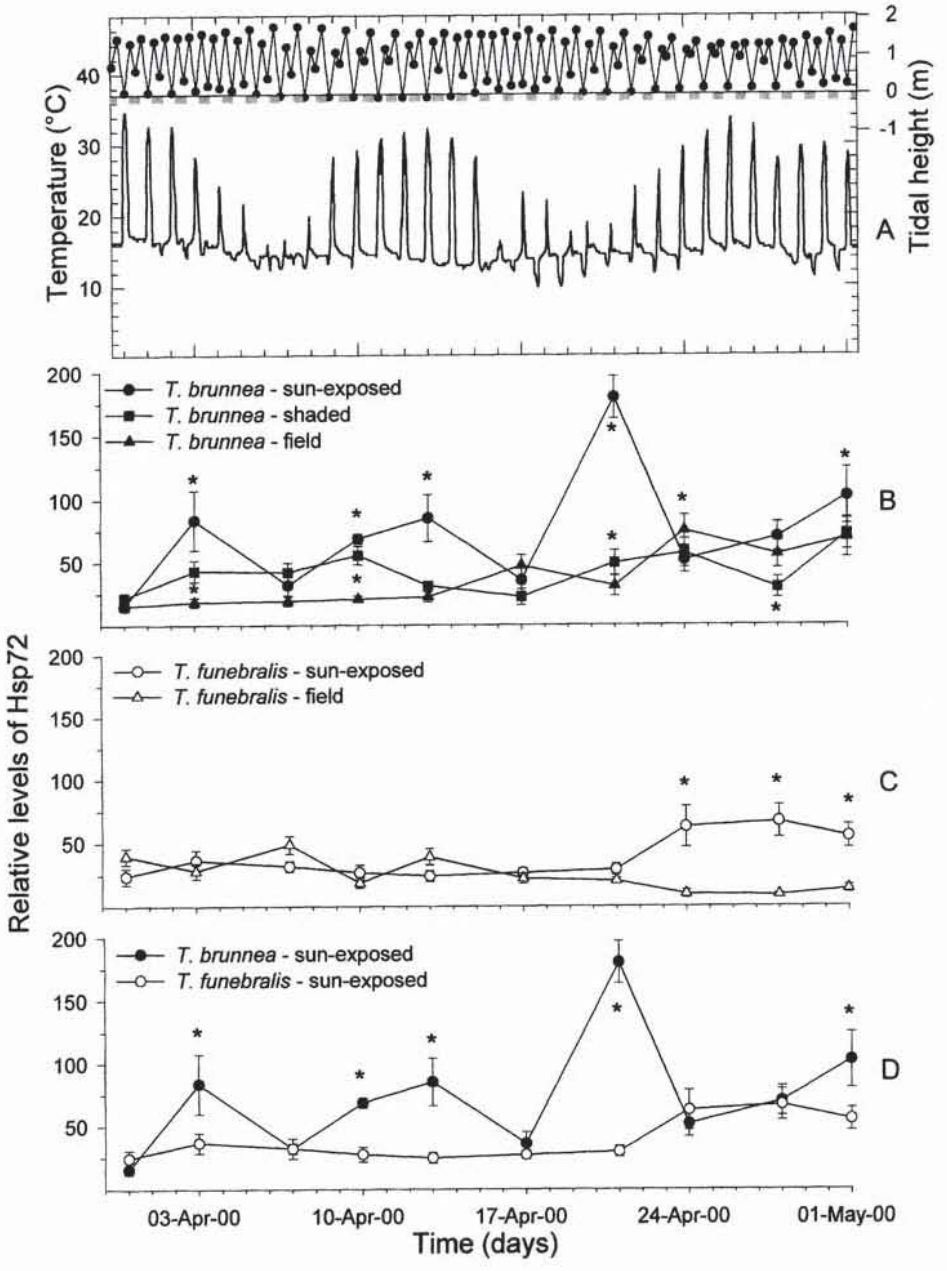

Figure 1. Environmental variation and time course of heat-shock protein expression (Hsp72) in experimental and control snails. (A) Tidal heights (m above mean lower low water) for Monterey Bay, day and night (gray) cycles, and temperatures recorded in a gelatin-filled snail shell (*Tegula funebralis* attached in the mid-intertidal zone). Snails within unshaded (sun-exposed) and shaded cages experienced lower temperatures. (B) Time course of endogenous levels of Hsp72 for specimens of *Tegula brunnea* transplanted to unshaded and shaded mid-intertidal cages *versus* field-acclimatized conspecifics (shallow subtidal zone). (C) Hsp72 levels for restricted (caged) and unrestricted (field-acclimatized) individuals of *T. funebralis* (mid-intertidal zone) and (D) for *T. funebralis* and *T. brunnea* individuals transplanted into unshaded mid-intertidal cages. Levels are expressed relative to an internal control (a bovine heat-shock cognate 70). Values are mean ± 1 SEM; * indicates significant differences ($P \leq 0.05$) among treatments; n = 5–7 snails for all data points (except n = 4 for sun-exposed *T. brunnea* on 21 April).

regime, consistently elevated in *T. brunnea* (with one exception on 7 April), supporting our previous results from laboratory acclimation experiments (Tomanek and Somero, 2002).

Discussion

Although temporal variation in levels of heat-shock protein (Hsp) has been suggested to closely track sublethal

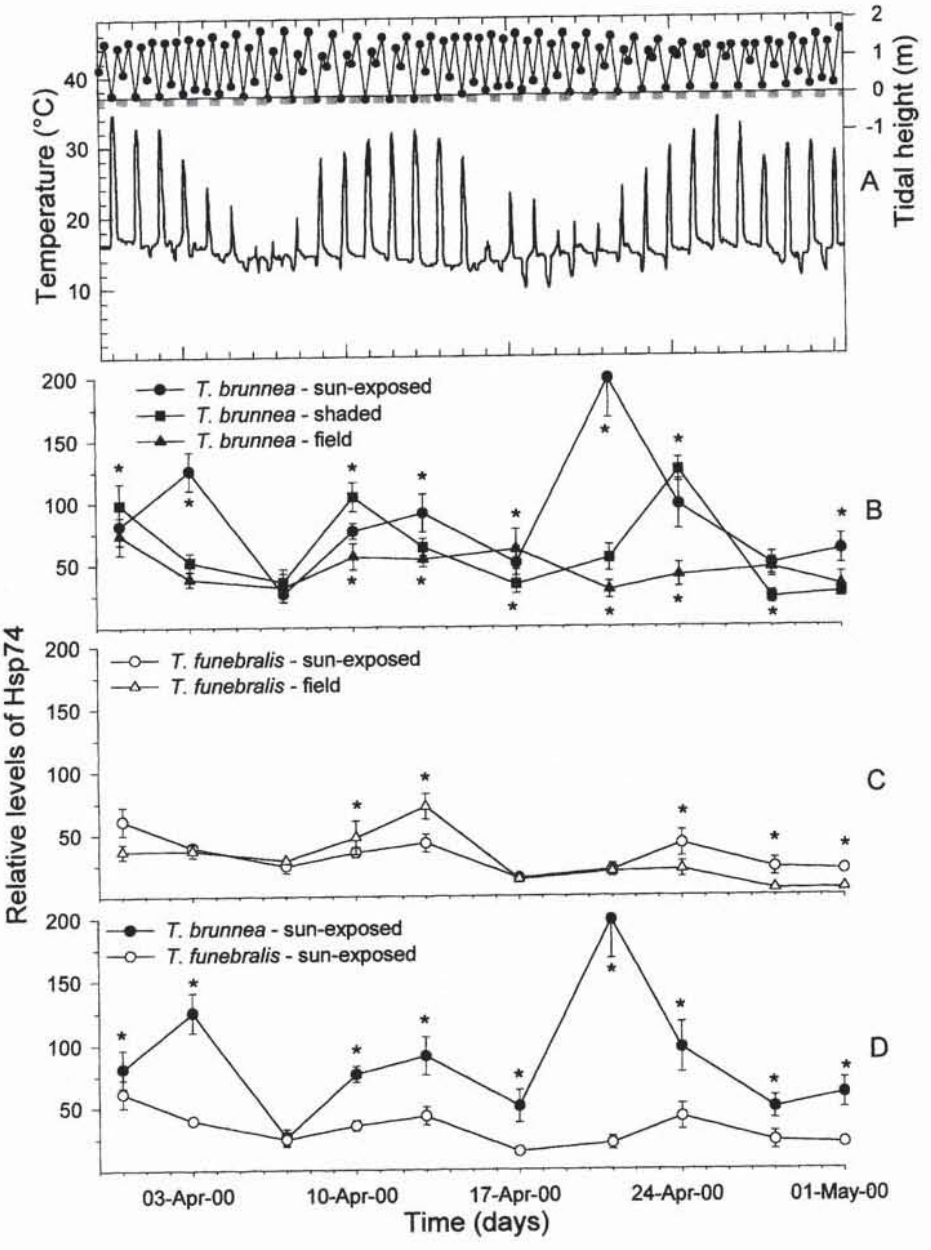

Figure 2. Environmental variation and time course of heat-shock protein expression (Hsp74) in experimental and control snails. (A) Tide heights, day and night cycles, and mid-intertidal temperatures (see Fig. 1 legend for details). (B) Time course of endogenous levels of Hsp74 for specimens of *Tegula brunnea* transplanted to unshaded (sun-exposed) and shaded mid-intertidal cages *versus* field-acclimatized conspecifics (shallow subtidal zone). (C) Hsp74 levels for restricted (caged) and unrestricted (field-acclimatized) individuals of *Tegula funebralis* (mid-intertidal zone) and (D) for *T. funebralis* and *T. brunnea* transplanted into unshaded mid-intertidal cages (see Fig. 1 legend for details).

stress, there have been few tests of this hypothesis under natural conditions. Furthermore, the ecological importance of interspecific variation in the heat-shock response deduced from laboratory studies has not been tested in the field. In this study we show that the subtidal to low-intertidal *Tegula brunnea* transplanted above its natural zone of occurrence experienced sublethal thermal stress, as reflected by elevated levels of two isoforms of the 70-kDa family of heat-shock proteins (Hsp72 and Hsp74). In contrast, levels of Hsp72 and Hsp74 varied little in control specimens of *Tegula brunnea* collected from the shallow subtidal zone, and less in *T. brunnea* individuals transplanted to shaded mid-intertidal cages. In addition, *T. funebralis* transplanted within its natural zone of occurrence, to mid-intertidal cages, but not field-acclimatized individuals, showed slightly elevated Hsp levels during a prolonged midday

low-tide period only. Here we discuss (1) how the time course of Hsp expression correlates with an organism's thermal history, and (2) how interspecific variation in the heat-shock response may limit the vertical distribution range of intertidal invertebrates.

Time course of Hsp levels

One of the objectives of this study was to examine the correlation between thermal events and the organism's response through time to better interpret the role of Hsps as biochemical indicators of sublethal thermal stress. The time course of levels of Hsp70 shows that periods of extreme thermal conditions upregulate endogenous levels of Hsp70 isoforms in transplanted *T. brunnea,* but not in individuals of both species that were collected from their natural thermal environment (Figs. 1B–C and 2B–C). One exception to the association between increased Hsp levels in transplanted *T. brunnea* and peak temperatures occurred on 21 April. However, changes in the daily temperature range (Tomanek, 2002) during the preceding days, from 16 to 17 April, were of similar magnitude as at the onset of a midday low-tide series (*e.g.,* 8 to 9 April), even if maximal temperatures were relatively low. Hsp levels were upregulated in response to physiological stress for not more than 3 to 4 days.

It is still unclear what thermal signals elicit an increase in Hsp levels. Levels in field-acclimatized mid-intertidal *T. funebralis* did not correlate with any of the thermal variables tested. Hsp levels will respond differently to chronic and acute thermal stress (Helmuth and Hofmann, 2001), but further studies are needed to test the response of Hsps to more complex thermal signals.

We predicted increased Hsp levels in transplanted *T. brunnea* on the basis of its long recovery ($>$ 50 h) in the laboratory from a heat-shock-inducing thermal exposure typical of the mid-intertidal zone (30 °C; Tomanek and Somero, 2000). These studies also indicated that *T. funebralis* would activate Hsp synthesis for at least several hours ($\leq$ 6 h for Hsp70) in response to temperatures of 30 °C or above—exposures that were reached at least several times during this month-long field experiment (Fig. 1A). In addition, the activation temperature of Hsp synthesis is 27 °C in laboratory-acclimated (constant temperature) specimens of *T. funebralis* that were exposed to a wide range of incubation temperatures following rapid heating in seawater (Tomanek and Somero, 1999). Although these laboratory conditions do not match field conditions completely, the activation temperature should be within a few degrees of 27 °C, certainly below 35 °C—one of the highest temperatures that field-acclimatized specimens of *T. funebralis* experienced during our month-long sampling period. Additionally, Hsp synthesis is upregulated for several hours in response to an acute thermal stress in mussels collected from the field after low tide, presumably in response to protein denaturation due to thermal stress (Hofmann and Somero, 1996b).

This discrepancy between our field results and our laboratory-based predictions could be due to several factors: first, most of our laboratory incubations were done in water, leading to a high rate of heating. However, heat stress in the intertidal zone is typically experienced under aerial conditions, when heating is slower (Tomanek and Somero, 2000). Second, our collection interval of 3 to 4 days may have missed an increase in levels of Hsp70 isoforms in response to thermal stress on some collecting days that followed several midday low tides after which individuals may show an attenuated response. Yet some of those collecting days were preceded by the "first" extreme low tide following several days of minor tides (*e.g.,* 10 and 24 April). Although the recovery time of elevated Hsp synthesis in response to heat stress in *T. funebralis* is short (6 h; Tomanek and Somero, 2000), we should have detected elevated endogenous Hsp levels over a much longer time period. Other studies have observed elevated Hsp levels in response to short-term acute severe heat stress (elevation lasting up to 2 weeks; Clegg *et al.,* 1998) and to long-term chronic mild heat stress (elevation as long as 4 days; Nakano and Iwama, 2002). Alternatively, Hsp70 is closely regulated and quickly eliminated in granules following heat shock (Morimoto, 1998), and we may have therefore missed briefly elevated levels of Hsp70. A final factor may be that the moderation of temperatures generated by the cages themselves (even those cages without additional shading), may have kept the temperatures of caged snails below 30 °C.

Interspecific variation in the heat-shock response and vertical distribution limits in intertidal invertebrates

By transplanting *T. brunnea* above its natural zone of occurrence and by following the time course of changes in endogenous levels of Hsps over a month of thermal variation, we were able to directly evaluate the importance of interspecific variation in the heat-shock response in relation to vertical distribution limits.

Transplanted specimens of *T. brunnea* increased their endogenous levels of both Hsp72 and Hsp74 in response to midday low-tide periods and therefore reached, as predicted, temperatures above the activation threshold for their stress response (Tomanek and Somero, 1999, 2000). Mortality in transplanted *T. brunnea* is also indicative of severe stress (8.5% mortality in sun-exposed individuals over the entire month; no individuals of *T. funebralis* died). Endogenous levels of Hsp72 and Hsp74 changed little in *T. funebralis* in comparison to *T. brunnea,* and this could be mainly due to this species' higher activation temperature. In the laboratory, *T. funebralis* activates the heat-shock response at 27 °C *versus* 24 °C in *T. brunnea* (following

acclimation to 13 °C and after rapid heating in seawater to a wide range of incubation temperatures). Thus the relative differences between activation temperatures of the stress response are good predictors of the relative levels of sublethal thermal stress and therefore the relative increases in endogenous levels of Hsps under common garden conditions, although the actual activation of the stress response depends on the heating rate and the medium (air *versus* water; Tomanek and Somero, 2000).

An increase in endogenous levels of Hsps in *T. brunnea* could also have been caused by the reduction in feeding time that accompanied the transplantation from the low- into the mid-intertidal zone. A reduction in feeding time is likely to lower metabolic rates (Shick, 1981; Branch *et al.,* 1988) and cellular energy levels (*e.g.,* ATP), which may disrupt protein homeostasis. Individuals of *T. brunnea* transplanted to shaded mid-intertidal cages differed from unmanipulated snails collected from the shallow subtidal zone in experiencing slightly higher body temperatures and much longer emersion times. Yet levels of Hsp72 and Hsp74 did not differ consistently between shaded mid-intertidal transplants and shallow subtidal controls, suggesting that longer emersion times *per se* were not activating increased Hsp levels. Other stress factors, *e.g.,* osmotic and desiccation stress, may also contribute to changes in Hsp70 levels, but these were not addressed in this study.

These results suggest that thermal conditions in the mid-intertidal zone are stressful for the subtidal to low-intertidal *T. brunnea,* but not for the low- to mid-intertidal *T. funebralis,* and thus may contribute to preventing *T. brunnea* from inhabiting the mid-intertidal zone. This is in large part due to the lower activation temperature (T_{on}) of the stress response, the 6 °C lower temperature of maximal Hsp synthesis (T_{peak}), and the lower temperature at which the synthesis of proteins (including Hsps) ceases in *T. brunnea* (T_{off}; Tomanek and Somero, 1999). In addition, levels of the heat-shock transcription factor1 (HSF1) are lower in *T. brunnea* than in *T. funebralis* (Tomanek and Somero, 2002).

T. funebralis is therefore better adapted to the physical conditions of the mid-intertidal zone, but such adaptations in the heat-shock response may be costly. For example, higher Hsp70 levels due to experimentally higher gene copy numbers of Hsp70 can impact life-history traits (*e.g.,* mortality and developmental time) that determine fecundity in *Drosophila* (Krebs and Feder, 1997a, b), and yeast strains with lower levels of Hsp104 grow faster (Sanchez *et al.,* 1992). Furthermore, Hsps can interact in detrimental ways with native proteins under nonstressful conditions and are therefore rapidly sequestered from the cytoplasm (Feder *et al.,* 1992). Thus, higher costs due to adaptations in the stress response to the mid-intertidal environment may in part explain why *T. funebralis* shows slower growth rates than its low-intertidal to subtidal congeners *T. brunnea* and *T. montereyi* (Frank, 1965; Paine, 1969; Watanabe, 1982).

However costly elevated levels of Hsps are, the transient upregulation of endogenous levels in subtidal to low-intertidal gastropods in the mid-intertidal zone shows that Hsps are good indicators of the thermal sensitivities of physiological systems under common field conditions. Our results also confirm our prediction that interspecific variation in the heat-shock response of *Tegula* congeners is adaptive to life in the thermally variable mid-intertidal zone (Tomanek, 2002).

Acknowledgments

The study was supported by the David and Lucile Packard Foundation and a National Science Foundation grant (IBN-0113184 to Dr. George Somero). We thank Michelle Phillips and Shanshan Bao for their help with the immunochemical analysis; Josh Troll for his help collecting animals in the field; and Drs. Jim Watanabe, Mark Denny, and Chris Harley for their comments and advice on the manuscript and statistical analyses. Dr. George Somero generously supported our work with advice, laboratory space, and funding. This is contribution number 137 of the Partnership for Interdisciplinary Studies of Coastal Oceans (PISCO): A Long-Term Ecological Consortium funded by the David and Lucile Packard Foundation.

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